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Tumor Necrosis Factor-α Is Undetectable in the Plasma of SS Patients With Elevated Hb F

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Steady-state sickle cell disease (SCD) patients may have increased plasma levels of acute phase reactants and pro-inflammatory cytokines because of subclinical inflammation. We have estimated TNF-α levels in the plasma and in supernatants following peripheral blood mononuclear cell (PBMC) activation with phytohemagglutinin (PHA) in a group of Kuwaiti SCD patients using ELISA. The group consisted of 28 SS, 8 Sβ-thal, and 2 SD patients all in steady state; 5 SS patients were studied during 7 episodes of painful crisis. The subjects were aged 2 to 16 years, with a mean of 7.3 ± 3.5 years. The β^S-globin gene cluster haplotype, α-tha1 status, and spleen function were determined in the SS group using standard techniques. Most (82%) were homozygous for the Saudi Arabia/India haplotype and had elevated Hb F levels ranging from 15% to 35%. There were 24 controls (Hb AA or AS), of whom 14 were healthy and 10 were acutely ill at the time of the study. None of the children with SCD (either in steady state or crisis) had detectable plasma TNF-α, but four controls (3 acutely ill and one healthy) had levels ranging from 61.7 to 249.8 pg/mL. Following PHA stimulation most subjects responded with high levels of TNF-α, with the median level among the steady-state SS patients being significantly higher than that in the controls (both the acutely ill or healthy). It therefore appears that because of the mild disease among our Arab SS children, TNF-α is not detectable in their plasma in steady state; these children, however, had a significantly higher response than controls following PBMC activation. Am. J. Hematol. 64:91–94, 2000.

Key words: tumor necrosis factor; sickle cell disease; elevated Hb F

INTRODUCTION

Sickle cell disease (SCD) is characterized by chronic hemolysis and recurrent occlusion of the microvasculature. While these processes are exacerbated during crisis periods, there is evidence of subclinical inflammation even in steady state. The metabolic abnormalities that have been reported in asymptomatic SS patients include doubling of protein turnover, increased C-reactive protein, serum amyloid A, and serum leukotriene C4 [1–4]. These suggest ischemic injury with hepatic synthesis of acute phase reactants and possible release of pro-inflammatory cytokines. Indeed several investigators have reported the presence of IL-1 and TNF-α in the plasma of American SS patients both in steady state and during crisis [5,6].

Sickle cell anemia is not uniform in its severity; several factors are known to ameliorate its presentation [7–9]. Gulf Arab patients, in particular, are known to have a mild clinical course probably due to their elevated Hb F levels and a high prevalence of co-existent α-thal trait [10,11].

TNF-α, a pleiotropic cytokine with a wide range of activities, has been implicated in the pathogenesis of several acute and chronic disease states, including sepsis, chronic infections and inflammatory conditions. It has potent effects on the synthesis of acute phase proteins by the liver and the expression of adhesion molecules on the vascular endothelium [12]. The present study was designed to investigate TNF-α levels in SCD patients (in steady state and crisis) in Kuwait. The influences of Hb

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F, co-existent α-tha1 trait, and spleen function were also investigated. The values were compared to those obtained from a group of normal controls.

PATIENTS AND METHODS

Patients

The SCD patients were attending the Pediatric Hematology clinics of Mubarak Al-Kabeer and Al-Amiri Hospitals in Kuwait. They were studied in "steady state," for example, no acute illness, crisis, or infection within 6 weeks of the study. Some patients were studied when they had vaso-occlusive crisis. There were two groups of controls: one made up of healthy siblings of the sickle cell patients and the other of children admitted to the children's ward of Mubarak Hospital with acute illnesses including infections.

Spleen function was assessed in the SS patients using ^{99}Tc-labeled tin colloid scintigraphy (Mallinckrodt Diagnostica, Holland) [13]. It was graded as normal, partial, or absent depending on how well the spleen was visualized in comparison to the liver.

About 5 mL of blood was obtained by venipuncture into vacutainers with EDTA anticoagulant. Complete blood counts were obtained with an electronic cell counter (Coulter S). A fresh hemolyzate was prepared from each sample and subjected to cation-exchange high-performance liquid chromatography (HPLC) to confirm the Hb genotype [14,15].

DNA was extracted from the leukocytes of the SS patients [16]. The β^S-globin gene cluster haplotypes were determined using allele-specific oligonucleotide hybridization with techniques previously described [17,18]. They were also screened for the deletional ($-\alpha^{3.7}$) α-thal-2 trait (which is the most common α-thal determinant in this community), using a PCR-based method [19].

Peripheral blood mononuclear cells (PBMC) obtained from the subjects by Ficoll-hypaque density gradient centrifugation were used for the induction of T cell proliferation with PHA. PBMC (10^5 cells per well) were stimulated with PHA (4 μg/mL) in triplicate. Twenty-four hours later, supernatants were collected for TNF-α estimation and a set of triplicate wells were pulsed with 1 μCi/well of (^{3}H)thymidine for 18 h, after which the cells were harvested onto glass fiber filters and the radioactivity counted. Detection of TNF-α in plasma and in culture supernatants was done using standard commercial ELISA immunoassay kits (Biosource International) [20,21]. The absorbance was measured at 450 nm in a Labsystems Multiskan MS reader. The concentrations of TNF-α in the test samples were determined from a standard curve (ranging from 0 to 1000 pg/mL) generated from standards supplied with the kit. The values are presented as medians (and ranges) except where otherwise stated; non-parametric testing was done using the Mann–Whitney *U* test. Where appropriate, the χ^2 test was used to determine the statistical significance of differences between proportions.

RESULTS

A total of 38 sickle cell disease patients in steady state were studied. They were made up of 28 SS, 8 Sβ-Thal, and 2 Hb SD patients. Five SS patients were studied during 7 episodes of vaso-occlusive crisis. The patients ranged from 2 to 16 years of age, with a mean of 7.3 ± 3.5 years.

The controls were made up of 21 Hb AA and 3 Hb AS individuals, 11 males and 13 females with a mean age of 7.8 ± 2.7 years; 14 were healthy at the time of the study, while 10 were acutely ill. Among the latter, 8 had respiratory infections of varying degrees of severity, 1 had acute gastroenteritis, and 1 had acute hemolysis from G6PD deficiency.

Twenty-three (82.1%) of the steady-state SS were patients were homozygous for the Saudi Arabia/India (SAI) haplotype, 3 (10.7%) were Benin/SAI compound heterozygotes, while 1 (3.6%) each was Benin/Benin and Bantu/Bantu. The range of Hb F in the SAI homozygotes was 15–35% with a mean of 22.3%; the three SAI/Benin patients had Hb F of 28, 11.4, and 9% respectively. The 2 patients with the other haplotypes had Hb F < 2%, and they also had the most severe clinical courses. The 5 SS patients studied during crisis episodes were SAI homozygotes.

The α-thal status was successfully determined in 24 of the SS patients in the study. Of these, 12 (50.0%) had the normal complement of four α-globin genes (αα/αα), 11 (45.8%) had one gene deletion (−α/αα), and 1 (4.2%) had two gene deletions (−α/−α).

^{99}Tc-labeled tin colloid liver/spleen scan was carried out in 20 SS patients. Of these, the spleen was visualized as clearly as the liver was in 9 (45.0%) individuals, it was partially visualized in 4 (20%), and it was not visualized at all in 7 (35%).

None of the steady-state SCD children had detectable plasma levels of TNF-α (Table I) while 4 (16.7%) of the controls had detectable levels ranging from 61.9 to 249.8 pg/mL. Three of the latter were acutely ill, two with pneumonia and one with acute hemolysis from G6PD deficiency. No further information is available on the apparently healthy control with detectable plasma TNF-α. None of the SS patients in crisis had detectable plasma levels.

PHA stimulation elicited an impressive response in terms of TNF-α production in most of the subjects studied (Tables I and II). The median value among the steady-state SS patients was significantly higher than in the controls (mean ranks of 29.87 and 19.93, respec-

TABLE I. TNF-α (pg/mL) Levels in Patients and Controls

	SS in steady state		SS in crisis		Sβ-Thal		Controls	
	Range	Median	Range	Median	Range	Median	Range	Median
Plasma	0–42.3	0	0	0	0	0	0–249.8	0
Culture	0–180.5	0	0–127.3	0	0–135.8	15.0	0–189.8	0
Stimulated	0–941.3	347.6	90.5–668.5	341.6	78.6–410.0	230.7	0–956.0	132.6

TABLE II. TNF-α (pg/mL) Levels in SS Subgroups

	SS + α-Thal[a]		SS − α-Thal[b]		SS + Spleen[c]		SS − Spleen[d]	
	Range	Median	Range	Median	Range	Median	Range	Median
Plasma	0	0	0	0	0	0	0	0
Culture	0–127.3	0	0–180.5	0	0–127.3	0	0–23.3	0
Stimulated	38.4–760.5	304.2	78.6–941.3	387.2	0–941.3	304.2	42.8–666.1	390.2

[a]SS patients with co-existent α-thal trait.
[b]SS patients without α-thal.
[c]SS patients with normal or partial spleen function.
[d]SS patients with no spleen function.

tively, z of -2.39 and $P = 0.017$). The post-activation TNF-α concentrations were stratified (<100 and ≥100 pg/mL) in each of the groups studied. Twenty-four (85.7%) of the SS patients had values ≥100 pg/mL compared to 11 (45.8%) of the controls; the difference in this distribution is statistically significant (with χ^2 of 9.3 and $P < 0.01$); the level of significance did not change when comparisons were made between the SS patients and either the acutely ill or healthy controls. Co-existent α-thal, gender, crisis, or spleen function status did not influence the level of response to PHA stimulation among the SS patients (Table II).

DISCUSSION

Gulf Arab SS patients with the SAI haplotype have elevated Hb F, which does not co-polymerize with Hb S and offers relative protection from intravascular sickling which is the basis of most of the complications of the disease [10,22]. In the present study, most of the patients were homozygotes for this haplotype and had Hb F levels ranging from 15% to 35%. The other ameliorating factor in our patients is the high prevalence of α-thal trait, which was found in about 50% of the cases in the present study. Previous studies from our center showed that SS patients with co-inherited α-thal trait were more likely to retain good spleen function, have a reduced incidence of infections [23] and reduced prevalence of gallstones probably because of a less severe chronic hemolysis [24] when compared to SS patients with low Hb F levels.

As far as we know, the present study is the first to estimate plasma TNF-α in SS patients with elevated Hb F levels. It is interesting that none of the SCD children had detectable plasma levels. To ensure that this was not due to poor sensitivity of the kit used, the assays were repeated with a kit from a different manufacturer (Immunotech) and the results were virtually identical. Among the SS children there were two (aged 2 and 3 years, respectively) with low Hb F (<0.2%), who were homozygotes for the Benin and the Bantu haplotype respectively. Although both have had severe clinical courses and been transfused previously, neither had detectable TNF-α in their plasma. It will be interesting to extend this study to adult Kuwaiti SS patients (both in steady state and in crisis) to determine how many may have detectable plasma TNF-α. We have recently tested a 35-year-old Kuwaiti steady-state SS patient who is a compound heterozygote for the SAI and Bantu haplotypes. She had a plasma TNF-α level of 42.3 pg/mL.

The possible pathogenetic role(s) of TNF-α in SCD remains to be fully elucidated. It has been postulated that the clinical and subclinical infections in SCD may continuously activate monocytes thus producing TNF-α even in steady state. Also, given the enhanced levels of C-reactive proteins and serum amyloid A in SS patients, it is tempting to speculate that pro-inflammatory cytokines such as TNF-α may be responsible for their production. The influence of TNF-α on endothelial cells may be relevant to the observed increase in adhesion of sickled erythrocytes to endothelial cells, probably contributing to increased disease severity [25,26].

Studies among steady state adult SS patients in Venezuela and the U.S. [5] and also among pediatric patients in Louisiana, U.S.A., have shown higher plasma levels of TNF-α in comparison to normal controls [6]. Moreover, the values were higher in those with a history of infections in the previous 2 years, but no clear relationship could be established with pain crisis or blood transfusion. In the present study, we found that none of the SCD children and only a few of the controls, had detectable

plasma levels of TNF-α but mitogen-activated PBMC from SS patients secreted significantly higher levels of TNF-α than from control subjects. Our observations probably reflect the in vivo situation in which activation of leukocytes (e.g., by bacterial antigens) would lead to higher levels of TNF-α in SS patients. Therefore, while our SCD children, on account of their relatively mild clinical course, may be unlikely to have detectable levels of plasma TNF-α in steady state, there is a need to extend these studies to patients with bacterial infections.

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